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*RECONSTITUTION OF IMMUNOLOGIC ACTIVITY BY INTERACTION OF POLYPEPTIDE CHAINS OF ANTIBODIES**

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In a previous publication[1] it was proposed that the capacity of antibodies to combine specifically with different antigens might result from interactions of separate polypeptide chains in the antibody molecule. 7S antibody molecules contain two types of polypeptide chains:[1-4] L (light) chains with molecular weights of approximately 20,000 and H (heavy) chains with molecular weights in the neighborhood of 60,000.[5,6] L chains are contained in the active fragments produced by hydrolysis of antibodies with papain.[4-7] The L chains of antibodies of different specificities differ in their patterns of separation by starch gel electrophoresis in urea.[8] Moreover, L chains are the common structural elements[4, 9] of the various classes of γ-globulins (γ, γ_{1A}, γ_{1M}), all of which are known to contain antibodies.

These observations prompted the suggestion that "similar or different L chains interact through weak forces with each other or singly with H chains to form the combining region."[1] The present communication describes some experimental results which support this hypothesis. Most of the experiments were performed using antibacteriophage antibodies since the assay of phage neutralization measures activity over a wide range with high sensitivity. After separation of the L and H chains of specifically purified guinea pig antibodies directed against f1 bacteriophage and f2 bacteriophage, the neutralizing activity of the chains declined to low levels. Mixing the L and H chains resulted in partial restoration of activity. Confirmatory data were obtained using antibodies directed against the dinitrophenyl (DNP) group, measuring the binding of this hapten by equilibrium dialysis. Similar results on the reconstitution of equine antibodies have been reported recently by Franěk and Nezlin.[10,11]

Materials and Methods.—Proteins, antigens, and immunization procedures: Pure f1 bacteriophage was obtained as described by Zinder *et al.*[12] The methods of isolation of pure f2 bacteriophage have also been described.[13] Guinea pig γ-globulin and rabbit γ-globulin were isolated by zone electrophoresis of serum on starch.[14] Dinitrophenyl-bovine serum albumin was prepared using the method of Farah *et al.*[15] The procedure of immunizing the guinea pigs has been detailed:[8] Each animal received initial injections in the footpads of a total of 0.4 mg of bacteriophage in

complete Freund's adjuvant. Subsequent intradermal injections of 0.4 mg of bacteriophage were given weekly in the skin of the back.

Isolation of antibodies: Except as noted, the antibodies were obtained from the sera of individual guinea pigs. Anti-DNP antibodies were isolated as described previously.[8] Purified antibodies directed against f2 phage were isolated using the method of Singer *et al.*[16] The same method was employed to purify the antibodies to rabbit γ-globulin. The following procedure was used to purify antibodies to f1 phage: immune precipitates formed near equivalence were washed three times with phosphate buffer, pH 7.0, $\Gamma/2$, 0.2. They were then treated with glycine sulfate buffer, pH 2.4, $\Gamma/2$, 0.35 to dissociate the complexes, and the preparations were centrifuged for 90 min at 78,000 $\times$ g to remove the phage. The supernate was neutralized with phosphate buffer, pH 7.0, $\Gamma/2$, 1.0, and dialyzed against 0.15 N NaCl. After centrifugation to remove the small amount of precipitate formed, the preparation was concentrated by ultrafiltration.

Tests for the purity of the antibody preparations included immunoelectrophoresis and double diffusion in agar which were performed as previously described.[4]

Reduction and alkylation of the purified antibodies and separation of L and H chains: Reduction and alkylation followed the description of Edelman and Poulik.[2] 1/2 % protein solutions were reduced for 2 hr at room temperature in phosphate buffer, pH 7.0, $\Gamma/2$, 0.04 made 0.1 M in 2-mercaptoethanol. The reaction mixtures were made 0.2 M in iodoacetamide, and after 10 min at room temperature they were dialyzed against 0.5 M propionic acid.

Separation of the chains followed the procedure of Fleischman *et al.*[17] Five to 10 mg of reduced alkylated antibody was filtered through 100 $\times$ 1.5 cm or 100 $\times$ 1 cm columns of Sephadex G-100 in 0.5 N propionic acid. The sample was applied in 1.0–1.5 ml volumes, the flow rate was 4.0–8.0 ml per hour, and 2.0 ml fractions were collected. The eluate was monitored by measuring absorbancy at 280 mμ. The purity of the fractions was tested by starch gel electrophoresis in urea.[2] Selected fractions obtained by gel filtration were pooled while still in propionic acid and dialyzed at 4°C against several changes of phosphate buffer, pH 7.0, $\Gamma/2$, 0.04 made 0.15 N in NaCl. In most of the mixing experiments, the L and H chain fractions were pooled in the ratio of the yield of absorbancy units given by the gel filtration patterns. When L or H chain fractions of two different antibody preparations were mixed, all of the dissociation and separation procedures were carried out simultaneously.

For clarity in presentation, the original specificity of the antibodies from which the chains are derived is denoted in parentheses next to the designation of the chain type, e.g., L(f1) or H(f1). Homologous mixtures are denoted: L(f1) + H(f1). Hybrid mixtures are denoted: L(f1) + H(f2) or L(f2) + H(f1).

Assay of bacteriophage neutralization: This assay method has been described in detail.[18] The assumption is that, in antibody excess, phage inactivation follows the relationship: $-\ln (p/p_0) = Kct$. p_0 is the plaque count at zero time; p is the plaque count at t min; c is the antibody concentration in units of absorbancy at 280 mμ, and K, the rate constant, is expressed per minute per unit absorbancy at 280 mμ. f1 and f2 phage neutralization was found to obey this relation over several decades of neutralization (see Fig. 3). Phage concentrations and dilutions were chosen so that at any point in the determination at least 200 plaques of residual phage could be counted in each replicate plate. This high plaque number diminishes the sampling error.

Equilibrium dialysis: One to 2.5 mg of the protein in 2.5 ml of 0.15 N NaCl were placed in one chamber of the apparatus. To the chamber on the opposite side of the membrane (Visking 23/32 casing) 2.5 ml of 0.15 N NaCl was added. 250 μl of 3.9 $\times$ 10^{-5} M C^{14} dinitrophenol (specific activity 1μc/μM) was added to each chamber and the apparatus was placed at 4°C for 48 hr without stirring. 100 μl aliquots from each compartment were counted in a liquid scintillation counter, and 24 hr later additional 100 μl aliquots were counted to ensure that equilibrium had been reached. The fluid from the compartment containing only hapten was replaced by 2.5 ml of 0.15 N NaCl and after 72 hr at 4°C, 250 μl aliquots from each chamber were counted. This procedure was repeated one more time. The data were plotted as difference in counts per minute across the membrane against the ratio between this difference and the counts per minute on the side containing only hapten. After extrapolation to infinite hapten concentration, it was calculated that approximately 0.5 sites were occupied per mole of native antibody.

Results.—The purified antibodies directed against f1 bacteriophage and f2 bacteriophage were free of contaminating serum proteins. This was shown by im-

munoelectrophoretic analyses using rabbit antisera against whole guinea pig serum and against isolated guinea pig γ-globulin (Fig. 1). The immunologic and starch gel electrophoretic analyses indicated that the antibodies were of the 7S γ class.

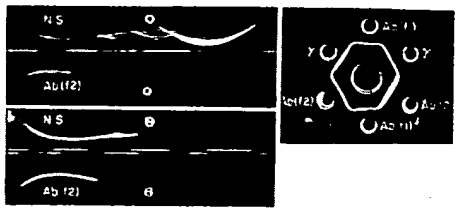

FIG. 1.—Immunoelectrophoresis and double diffusion in agar of preparations of purified guinea pig antibodies. (*a*) Immunoelectrophoresis using rabbit antiserum directed against guinea pig serum; (*b*) immunoelectrophoresis using rabbit antiserum directed against guinea pig γ-globulin; (*c*) double diffusion in agar. Central well contained rabbit antiserum directed against guinea pig serum. γ = guinea pig γ-globulin; Ab (f1) = antibodies against f1 phage; Ab (f2) = antibodies against f2 phage; N.S. = normal guinea pig serum.

Two patterns of separation of the chains of partially reduced alkylated antibodies were obtained after filtration. The first type (Fig. 2*A*) showed two incompletely resolved peaks followed by a well-resolved peak. The second type (Fig. 2*B*) showed no resolution of the first two peaks. Both types of patterns were found for different antibody preparations directed against f1 phage and f2 phage. Depending on the pattern obtained, the H chain fraction was taken either to be the second peak (Fig. 2*A*) or the trailing portion of the first peak (Fig. 2*B*). The L chain fraction consisted of the major portion of the well-resolved and most retarded peak in each case. As shown by starch gel electrophoresis in urea (Fig. 2*C*), this fraction appeared to be free of H chains and of undissociated material. The H chain fractions contained material of higher molecular weight which was also present in earlier fractions, regardless of the type of separation pattern.

Phage neutralization by anti-f1 antibodies, by the separated chain fractions, and by mixtures of the chain fractions is illustrated in Figure 3. Reduction and alkylation of the antibodies caused slight diminution of phage-neutralizing activity. A

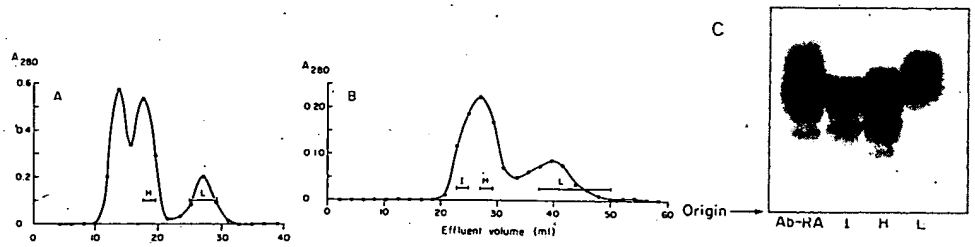

FIG. 2.—Separation of H and L chain fractions from reduced alkylated antibodies by gel filtration on Sephadex G-100 in 0.5 *N* propionic acid. (*A*) Reduced alkylated anti-f1 antibodies; (*B*) reduced alkylated anti-f2 antibodies; (*C*) starch gel electrophoresis of fractions from Fig. 2*B*. I = first fraction; H = H chain fraction; L = L chain fraction; Ab-RA = reduced alkylated antibodies; A_{280} = absorbancy at 280 mμ.

greater loss of neutralizing activity occurred when partially reduced alkylated antibody was exposed to 0.5 *N* propionic acid for the same time and at the same temperature as the sample that was subjected to gel filtration. L(f1) chains showed little or no neutralizing activity wheras H(f1) chain fractions showed low levels of activity. The mixture of chains showed enhancement of activity of about fourfold over that expected if there were no interaction.

Results of a similar experiment in which homologous and hybrid mixtures of chains from anti-f1 and anti-f2 antibodies were compared are given in Table 1. At the levels tested, there was no cross-reactivity of the two antibody systems or of their separated chain fractions. In both systems, reduction and alkylation led to a drop in neutralizing activity. Reduced alkylated antibodies exposed to propionic acid showed a marked drop in activity. Both H(f1) and H(f2) chain fractions had some residual activity, whereas the L chains were minimally active in both cases.

TABLE 1

RECONSTITUTION OF ACTIVITY OF ANTIPHAGE ANTIBODIES

Sample	K (per minute per unit absorbancy at 280 mμ) f1 neutralization	f2 neutralization
Ab (f1)	5,300	0
Ab (f1) – RA*	1,900	0
Ab (f1) – RAP*	190	0
H (f1)	19	0
L (f1)	0.9	0
Ab (f2)	0	230
Ab (f2) – RA	0	140
Ab (f2) – RAP	0	58
H (f2)	0	9.3
L (f2)	0	1.9
H(f1) + L(f1)	53 (9.6)†	0
H(f1) + L(f2)	16 (9.6)	0.4
H(f2) + L(f1)	0.2	11 (4.6)
H(f2) + L(f2)	0	14 (4.6)

* Abbreviations: Ab = antibody (Ab (f1) and Ab (f2) were isolated from two different guinea pigs); RA = reduced alkylated; RAP = reduced and alkylated and exposed to propionic acid for the same length of time as the separated fractions.

† Numbers in parentheses refer to activity expected from the amount of H chain fraction present in the mixture.

Homologous mixtures of H(f1) and L(f1) fractions showed a fivefold enhancement of activity over that expected for the amount of H(f1) chains present in the mixture. H(f1) + L(f2) hybrid mixtures showed a twofold enhancement, whereas H(f2) + L(f1) hybrids were inactive against f1 bacteriophage. In the f2 system, H(f2) + L(f2) mixtures showed a threefold enhancement of activity; hybrid H(f2) + L(f1) mixtures showed a twofold enhancement. H(f2) + L(f2) and H(f1) + L(f1) mixtures were active only against the homologous antigens.

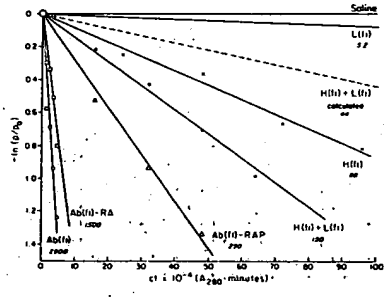

FIG. 3.—Phage neutralization by anti-f1 antibodies, chain fractions, and mixtures. Ab (f1) = antibody against f1 phage isolated from pooled sera of two animals; Ab (f1) RA = reduced alkylated antibody; Ab (f1) RAP = reduced alkylated antibody exposed to 0.5 *N* propionic acid; H (f1) = H chain fraction; L (f1) = L chain fraction. Numbers in italics refer to K, the rate constant expressed in dimensions of per minute per unit absorbancy at 280 mμ (see *Materials and Methods*). The measured points determining the line for L (f1) fall outside the graphed region and are not shown.

In a separate experiment, H(f2) + L(f2) mixtures were found to be ten times as effective in neutralization as mixtures of H(f2) with L chains from purified guinea pig antibodies directed against rabbit γ-globulin. Hybrid mixtures of H(f2) chain fractions with L chains of the nonspecific γ-globulin from the same animal were only one seventh as effective as homologous H(f2) + L(f2) mixtures.

Results consistent with those shown in Figure 3 and Table 1 were found upon measuring the phage-neutralizing activity of each tube obtained by gel filtration of reduced alkylated anti-f1 antibodies (Fig. 4). The highest activity was found in the material from the first peak. Less activity was found in the H chain fraction, and another small peak of activity was found in the region between the

H and L chain fractions. No activity was seen in the L chain fraction itself. The high activity in material from the first peak was correlated with the presence of incompletely dissociated antibodies as shown by starch gel electrophoresis. The region between the H and L fractions would be expected to contain both types of chains and therefore also the reconstituted activity.

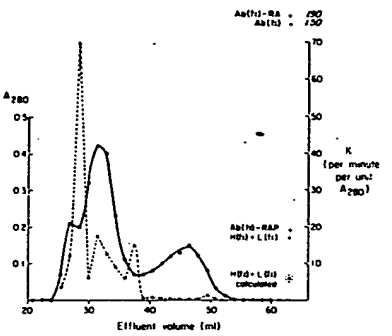

FIG. 4.—Activity in chain fractions of anti-f1 antibodies (Sephadex G-100 in 0.5 *N* propionic acid). O——O absorbancy at 280 mμ; ●——● activity in terms of K. On right are given the K values of the untreated antibodies Ab (f1), the reduced alkylated antibodies Ab (f1) RA, and the chain mixture H(f1) + L(f1). The antibodies were isolated from pooled sera of four animals.

In several other experiments phage neutralization was observed when reconstitution was attempted using antibodies of completely unrelated specificities as starting material. For example, L(f1) chains from certain preparations were found to neutralize f2 phage, and this neutralization was enhanced when either L(f1) + H(f1) or L(f1) + H(f2) mixtures were used. Furthermore, the mixture H (DNP) + L (DNP) showed measurable amounts of phage neutralization (Table 2), although the neutralization by Ab (DNP) and the H (DNP) and L (DNP) chains was at much lower levels. It should be stressed, however, that the neutralization of f2 phage by mixtures of chains from apparently unrelated antibodies was at lower levels than that of mixtures of the chains of homologous antibodies. Moreover, H chain fractions from nonspecific guinea pig γ-globulin showed low levels of neutralization ($K = 0.3$).

TABLE 2

f2 PHAGE NEUTRALIZATION BY MIXTURES OF CHAINS FROM ANTI-f2 AND ANTI-DNP ANTIBODIES

Sample	K (per minute per unit absorbancy at 280 mμ)	
Ab (f2)	860	
Ab (f2) − RAP*	3.2	
H (f2)	3.6	
L (f2)	0.17	
Ab (DNP)	0.05	
H (DNP)	0.13	
L (DNP)	0.13	
H(f2) + L(f2)	29.0	(1.8)†
H(f2) + L(f2) saline‡	6.5	(1.8)
H(f2) + L(DNP)	9.6	(1.9)
H(DNP) + L(f2)	0.08	(0.15)
H(DNP) + L(DNP)	3.1	(0.13)

* RAP = reduced and alkylated; exposed to 0.5 *N* propionic acid.
† Numbers in parentheses refer to activity expected from the amount of H chain fraction present in the mixture.
‡ Fractions were mixed after dialysis against 0.15 *N* NaCl + phosphate buffer pH 7.0, Γ/2, 0.04.

TABLE 3

BINDING OF C^{14} DINITROPHENOL BY ANTI-DNP ANTIBODIES AND THEIR POLYPEPTIDE CHAINS

Sample	cpm bound†
Ab	900
Ab − P*	790
Ab − RA*	710
Ab − RAP*	480
H(DNP)	380
L(DNP)	<200
H(DNP) + L(DNP)	580‡

* Abbreviations: P = exposed to 0.5 *N* propionic acid; RA = reduced and alkylated; RAP = reduced and alkylated and then exposed to 0.5 *N* propionic acid; Ab = antibody.
† cpm bound = counts per minute calculated to be bound per unit absorbancy of protein at 280 mμ after extrapolation to infinite hapten concentration.
‡ The maximal value expected if the contributions of H and L chains were only additive is 340 cpm per unit absorbancy at 280 mμ. The ratio of absorbancies of H chains to L chains in the mixture was 4:1.

As shown in Table 2, the mixture of H(f2) and L(f2) chains after dialysis against saline was less effective in phage neutralization than mixture in propionic acid followed by dialysis. Otherwise, the results were similar to those in Table 1.

Reconstitution of antibody activity in a system measuring binding directly was observed using purified anti-DNP antibodies. The results of an equilibrium dialysis experiment in which C^{14} dinitrophenol was the hapten are given in Table 3. Reduction and alkylation of the antibody or exposure of untreated antibody to 0.5 *N* propionic acid resulted in slight diminution of binding activity. As in the experiments utilizing antiphage antibodies, exposure of the reduced alkylated antibody to propionic acid resulted in a considerable drop in activity. The H chain fraction retained some binding affinity as did the L chain fraction. The H (DNP) + L (DNP) mixture showed an increase in affinity of 1.7 times that expected if the H (DNP) fraction and L (DNP) fraction were binding without interaction. The H (DNP) + L (DNP) mixture showed 64 per cent of the binding of untreated antibody. The native antibodies, chain fractions, and mixtures all had association constants of the same order of magnitude (approximately 10^6 l/mole).

Discussion.—The aim of the present study was to determine whether chain interaction influences the activity and specificity of antibodies. For this purpose, we chose three unrelated systems of purified guinea pig antibodies: anti-f1 phage, anti-f2 phage, and anti-DNP. The antibodies, their separated L and H chains, and homologous and hybrid mixtures of the chains were tested for activity by assay of phage neutralization and in some cases by equilibrium dialysis. Before discussing the implications of the experimental results, we wish to stress that the two assay methods may not test the same functions of antibodies. The assay of phage neutralization is a kinetic test which measures binding, but in addition may depend upon functions of the antibody molecule other than binding. Equilibrium dialysis measures only the binding of hapten to proteins under conditions of thermodynamic equilibrium. This method has the advantage of testing binding directly, whereas the neutralization assay has the advantage of measuring an enormous range of activities with great sensitivity.

As measured by both methods, specific antibody activity was partially restored by recombination of homologous H and L chain fractions, and these results strongly support the chain interaction hypothesis. Although only about 1–6 per cent of the original activity of native antiphage antibodies was recovered, the mixtures of H and L chain fractions were 2–10 times more active than any of the unmixed fractions. The level of activity reconstituted in the mixtures was approximately the same as the amount of activity remaining in reduced alkylated antibodies that had been exposed to 0.5 *N* propionic acid.

Considerably greater reconstitution was obtained in the DNP system, as measured by equilibrium dialysis. Up to 60 per cent of the original hapten-binding capacity was restored by mixing the L and H chain fractions. The difference in the degree of reconstitution of the antihapten antibodies and antiphage antibodies may result from differences in the heterogeneity of their polypeptide chains.[8] It may depend also upon the more stringent requirements of the phage assay methods, as emphasized above.

In the experiments on the antiphage antibodies, the purity of the L chain fractions was indicated by starch gel electrophoresis, as well as by the fact that they had no activity. On the other hand, starch gel electrophoresis of the H chain fractions showed the presence of small amounts of incompletely dissociated material. This material may be responsible for the residual activity found in H chain fractions,

in view of the relatively high activities found in material from the first peak of the filtration pattern shown in Figure 4. Although this material may still contain interchain disulfide bonds, the mixing experiments indicate that interchain disulfide bonds are not absolutely required for partial reconstitution of activity.

Homologous mixtures of chains (e.g., L(f1) + H(f1) using f1 phage as test antigen) were consistently more effective in reconstituting activity than hybrid mixtures (e.g., L(f1) + H(f2) or L(f2) + H(f1)). Most of the hybrid mixtures showed reconstitution only when H chains from antibodies originally directed against the test antigen were used. This was also observed by Franěk and Nezlin[10] who concluded that the antibody specificity is determined by the H chain but the presence of the L chain is required for the formation of a fully active combining site. This conclusion would be fully warranted if L chains of different origin were equally effective in reconstituting specific activity. The experiments revealed, however, that homologous L chains are more effective than heterologous L chains. This indicates that L chains also contribute to the specificity, a conclusion consistent with both the heterogeneity and the structural differences found among L chains of antibodies of different specificities.[1, 8]

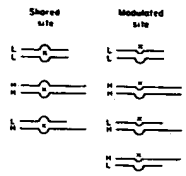

FIG. 5.—Hypothetical models of chain interaction (× marks the vicinity of one antibody combining site).

The activity and specificity of antibodies thus appears to be a complex function of the structure and interaction of both H and L chains. There are two different modes by which an active site might be generated through chain interaction, as illustrated diagramatically in Figure 5. Both types of chains might contribute amino acid residues directly to the site (shared site). On the other hand, interaction of one chain with another might result in formation of a stable binding region on only one of the chains (modulated site). In addition, both modes may operate together but to a different extent in different antibodies.

The diagrams shown in Figure 5 are based on the assumption that the 7S antibody molecule consists of two L and two H chains.[3–6] If the molecule consists of four L and two H chains, the same basic mechanisms of interaction could occur. The present experimental data exclude the possibility of shared or modulated sites exclusively between two H chains. Among the remaining possibilities, the most probable would seem to be shared or modulated interaction between L and H chains.

The type of experiment described here may not permit one to draw definite conclusions about the relative contributions of the L and H chains to the specificity. For example, a modulated site on an H chain[17] might have been generated by interaction with one particular type of L chain. Stabilization of the active conformation may then arise from intrachain interactions (including disulfide bonds). Subsequent experimental removal of the L chain would not necessarily destroy the active conformation completely. Since L chains have certain common structural features,[3, 4] interaction of the H chains with L chains of different origin might lead to different degrees of reconstitution.

Specific combining activity can result from interaction of more than one type of L chain with H chains derived from an antibody. This suggests that specific combining activity may arise from a variety of different chains. Several of the present observations indicate that such complex interactions are possible. Signifi-

cant levels of f2 phage neutralization were obtained with L (DNP) + H (DNP) mixtures, although the native anti-DNP antibody showed no neutralizing activity. In some experiments, L(f1) chains neutralized f2 phage, and the neutralization was enhanced by mixing L(f1) chains with either H (f1) or H(f2) chain fractions. In contrast, none of the chain fractions or mixtures from anti-f2 antibodies neutralized f1 phage. H chains from normal γ-globulins were also found to neutralize f2 phage. In all of these experiments the neutralizing activity was lower than that of homologous mixtures of chains from anti-f2 antibodies.

These observations suggest that γ-globulins and antibodies originally showing no specificity for certain test antigens can yield chains which interact to form complexes capable of binding to those antigens. Classical notions of immunologic specificity imply that the test antigen and the immunogen have the same or closely related structures. It is not known whether the noncross-reactive antigens used in the present study share any structural features. Moreover, the test antigen may alter the conformation of interacting L and H chains derived from antibodies originally unrelated to the test antigen. Alternatively, among any given set of L and H chains, a great variety of interactions may be possible, and some may be capable of binding selectively to certain antigens. Experiments to decide among the various possibilities are in progress.

Summary.—Dissociation of purified guinea pig antibodies against f1 phage, f2 phage, and the dinitrophenyl group into L and H polypeptide chains led to a marked drop in the activity of the separated fractions. Mixture of the L and H chain fractions of the same antibody preparation resulted in partial reconstitution of activity as measured by phage neutralization or equilibrium dialysis. Reconstitution of activity against a given antigen was also found using mixtures of H chains from antibodies originally directed against that antigen and L chains from unrelated antibodies or γ-globulin. In every case, however, the reconstitution of activity was greatest when both L and H chains from the homologous antibodies were mixed. Mixtures of L chain fractions from homologous antibodies and H chain fractions from unrelated antibodies usually showed no activity. In some cases, however, activity was observed when L and H chains from antibodies originally unrelated to the test antigen were mixed.

The results support the chain interaction hypothesis of antibody activity and suggest that both H and L chains contribute to immunologic specificity.

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THE ULTRAVIOLET PHOTOCHEMISTRY OF DEOXYURIDYLYL (3′→5′) DEOXYURIDINE

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The work of Wacker *et al.*[1] and Smith[2] has shown that, when uracil is irradiated with ultraviolet light (UVL), it is converted into two major photoproducts, viz., 5-hydro-6-hydroxyuracil (the hydrate) and dimers in which two uracil moieties are linked by a cyclobutane ring connecting their 5 and 6 carbon atoms. Grossman[3] has shown that, when polyuridylic acid is irradiated with UVL, its coding properties are altered when it is used as a messenger in an *in vitro* polypeptide synthesizing system. In this system it is not clear whether the observed effects are due to the production of uracil hydration products or dimers, or both. Investigating this question with polyuridylic acid is not easy. We have therefore studied the photochemistry of the model compound, deoxyuridylyl (3′→5′) deoxyuridine (dUpU), which we assumed would have similar photochemistry to UpU but which is much easier to synthesize. Wierzchowski and Shugar[4] investigated the photolysis of uracil dinucleotides and found effects which can now be understood in terms of the formation of intramolecular dimers as well as hydration products of uracil.

In the present paper the photoproducts produced on the irradiation of radioactive dUpU were separated by paper chromatography. Four major photoproducts were found, and the rates of production of these have been studied as a function of wavelength.

Materials and Methods.—Irradiations were carried out with a high intensity UV monochromator to be described in detail elsewhere. The instrument used as dispersing elements a water preprism between the source (BH6, Hg arc) and entrance slit, and a blazed grating (blaze wavelength 200 mμ) with dimensions 25.6 × 20.6 cm. The entrance and exit slits were 15 cm high and 0.6 cm wide, and the dispersion was 4 mμ per cm at the exit slit. Light from the exit slit was collected by an *f*/1.2 mirror and focused at the center of a stoppered quartz cuvette.

dUpU, labeled with P^{32} at an initial specific activity of approximately 10 mc/mmole, was prepared by condensation of 3′-O-acetyldeoxyuridine-5′-phosphate (made by a combination of the methods of Tener[5] and Smrt and Sorm[6]) with 5′-O-tritryldeoxyuridine[6] in the presence of dicyclohexylcarbodiimide (Gilham and

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*RECOVERY OF SPECIFIC ACTIVITY AFTER COMPLETE UNFOLDING AND REDUCTION OF AN ANTIBODY FRAGMENT**

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It has been shown in two previous papers from this laboratory[1, 2] that univalent active fragments of rabbit antibodies can be fully unfolded in concentrated guanidine hydrochloride with disruption of all noncovalent interactions. Upon removal of the unfolding agent, in the absence of antigen, the protein spontaneously returned to its native conformation, as measured by physicochemical criteria, and recovered 75 per cent of its ability to combine specifically with antigen. It was concluded that specific antibody activity cannot be generated by antigen-directed arrangement of noncovalent bonds.[3] These results suggested instead that antibody specificity arises in the same way as enzyme specificity, from specificity in the sequence of amino acids. It was pointed out, however, that definite proof of this contention required that specific antibody activity be recovered spontaneously after all disulfide bonds of the native protein, as well as noncovalent bonds, were disrupted. Recovery of activity after such treatment has been observed for a number of enzymes.[4–7]

The necessary proof has recently been provided in a paper by Haber.[8] He completely unfolded a univalent active fragment from rabbit antibody directed against ribonuclease, and cleaved all of its disulfide bonds by reduction. The protein was allowed to refold and reoxidize in the absence of antigen, and substantial re-

covery of the ability to combine specifically with ribonuclease was observed.

We wish to report similar results, using univalent active fragments from rabbit antibody directed against a haptenic group, the dinitrophenyl (DNP) group.

Materials and Methods.—Rabbit anti-DNP was prepared by the method of Farah *et al.*[9] Univalent active fragments (both type I and type II) were prepared from the antibody by the method of Porter.[10] A sample of fragment I was also prepared from nonspecific rabbit γ-globulin which had been fractionated from pooled rabbit sera. DNP-lysine was purchased from Mann Research Laboratories, Inc. Guanidine hydrochloride was prepared from Eastman guanidine carbonate by the method of Anson.[11] N-acetyl-L-tryptophanamide, used as reference standard for fluorescence measurements, was purchased from Cyclo Chemical Corp. Other chemicals were of reagent grade.

Protein concentrations were determined from measurements of absorbance at 278 mμ using a value of 1.50 for the absorbance of fragment I or fragment II at a concentration of 1 mg/ml in a 1-cm light path.

Reduction was carried out following the procedure of Crestfield *et al.*[12] with a few modifications, the substitution of guanidine hydrochloride for urea being the most important. The protein was exposed to 6 *M* guanidine hydrochloride, 1 mM EDTA, tris acetate buffer (pH 8.6, I = 0.1), and 0.1 *M* β-mercaptoethanol for 4 hr at room temperature. The protein concentration was usually about 0.4 mg/ml.

In order to demonstrate that this procedure reduces all disulfide bonds completely, one sample of reduced protein was S-carboxymethylated by adding 1.08 mmoles of iodoacetic acid in 1.0 ml of 1 *M* sodium hydroxide for each mmole of β-mercaptoethanol in the reducing medium. After 15 min at room temperature, the solution was dialyzed in the cold room against buffer at pH 8.6, and then at pH 5.5. The alkylated protein was hydrolyzed for 24 hr at 110°C in 6 *M* HCl, and subjected to amino acid analysis after the method of Spackman, Stein, and Moore.[13] Disulfide bonds which had not been reduced would have appeared in the analysis as half-cystine. As Table 1 shows, no trace of half-cystine was found. This experiment was carried out on a sample of fragment I from nonspecific γ-globulin, but it is assumed that the same result would be obtained with antibody fragment, since it is as readily unfolded by guanidine as is the nonspecific fragment,[2] and its disulfide bonds should thus be equally accessible to the reducing agent.

Reoxidation of reduced protein was carried out by cooling the solution to 5°C, and diluting it to make a solution in tris acetate buffer (pH 8.6, I = 0.05), 3 mM β-mercaptoethanol, 1 mM

TABLE 1

PARTIAL AMINO ACID COMPOSITION OF NATIVE AND REDUCED AND S-CARBOXYMETHYLATED FRAGMENT I*

	Residues per Mole†		
	Native Fragment I		After reduction and S-carboxymethylation
Amino acid	Mandy *et al.*[14]	This paper	This paper
S-carboxymethyl cysteine	—	0.6	15.9
Aspartic acid	34.6	33.4	33.3
Threonine	63.0	63.5	62.6
Serine	51.0	55.4	53.6
Glutamic acid	35.3	34.1	34.8
Proline	31.2	28.5	31.0
Glycine	42.5	40.9	41.7
Alanine	30.9	30.8	31.1
Half-cystine	(17.0)‡	15.3	0
Valine	43.7	42.7	43.1
Methionine	2.4	2.0	2.4
Isoleucine	12.6	12.7	12.9
Leucine	29.3	29.9	28.8
Tyrosine	20.1	22.4	21.9
Phenylalanine	13.3	14.5	13.8

* Digested 24 hr at 110°C in 6 *M* HCl. The comparable data from Mandy *et al.*[14] were obtained from a 22-hr digestion in the same medium.

† Basic amino acids and tryptophan were not determined. All figures were adjusted to give a total of 427 residues for the sum of all amino acids listed in the table.

‡ Half-cystine was not determined by Mandy *et al.* Analyses by Porter[10, 15] show that fragment II of rabbit γ-globulin has 13.6 and fragment I about 17 half-cystine residues per mole.

EDTA. The protein concentration was about 4 μg/ml. The solution was left in the cold for about 18 hr, and then slowly stirred at room temperature for about 20 hr. The solution was concentrated down to about one fifth the original volume by vacuum dialysis. It was then dialyzed against dilute tris acetate buffer, pH 8.6. The solution was further concentrated, dialyzed against sodium acetate buffer (pH 5.5, I = 0.05), heated briefly at 35–40° C, and clarified by centrifugation. It was found that about half of the original protein was recovered after this treatment. At least a part of the remainder was insoluble at pH 5.5, presumably because some polymeric species were created by formation of interchain disulfide bonds in place of some of the intrachain bonds present in the native protein.

Anti-DNP activity was determined by measuring the quenching of fluorescence which accompanies binding of the dinitrophenyl group. The procedure is based on that of Velick *et al.*,[16] and is described by Noelken and Tanford.[3] The antibody fragment was titrated with DNP-lysine, and the activity was calculated assuming that the fluorescence would be completely quenched if the antibody were 100% active.

Results.—It was first demonstrated, by measurement of sedimentation velocity and optical rotatory dispersion, that the reoxidized protein was closely similar to native fragment I by physical criteria. Immunological similarity was shown by precipitation of the reoxidized protein with goat antiserum against native rabbit fragment I. Mild reduction, followed by gel filtration in 1 *M* propionic acid, according to the procedure of Fleischman *et al.*,[15] gave a pattern similar to native protein, and showed that both A piece and B chain were present. Gel filtration in 1 *M* propionic acid without previous reduction showed that most of the product remained undissociated, demonstrating that the two chains of the reoxidized fragment were joined by disulfide bonds. These measurements were carried out on reoxidized nonspecific fragment only, because insufficient quantities of antibody fragment were available, and they will be described in detail at a later date.

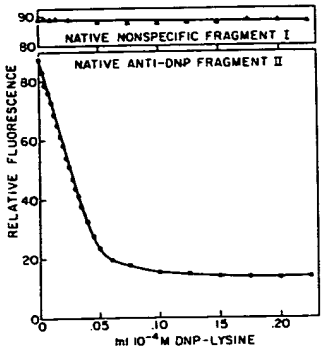

FIG. 1.—Titration of native antibody fragment II with DNP-lysine. The upper curve is from a control experiment using fragment I from nonspecific γ-globulin. Fluorescence intensities are reported relative to a standard solution of N-acetyl-L-tryptophanamide. Differences in the *initial* values of the relative intensity, here and in the following two figures, simply reflect differences in protein concentration.

Antibody activity was measured in terms of the per cent quenching of tryptophan fluorescence which could be attained by titration of the protein with DNP-lysine. Figure 1 shows the result of such a titration with native anti-DNP fragment II. It shows that 84 per cent of the fluorescence can be quenched, whereas no quenching at all occurs in the control experiment with a similar fragment from nonspecific γ-globulin. A similar curve was obtained with the anti-DNP fragment I used in this study, except that it was somewhat less active (72 %). The first portion of the quenching curve, for both native fragments, is steep and linear, and the slope shows that each molecule of DNP-lysine added quenches the entire fluorescence of an antibody fragment. Many of the fragment molecules must therefore possess

a very high binding constant for the specific hapten. However, some molecules with lesser affinity are also present, as shown by the curvature of the quenching curve as saturation is approached. This is the normal behavior for anti-DNP preparations.[17]

Unfolded and reduced antibody fragment was not tested for activity, since it has already been shown[2] that unfolding by guanidine hydrochloride alone, without scission of disulfide bonds, leads to complete loss of antibody activity.

When unfolded and reduced antibody fragment was allowed to refold and reoxidize, in the absence of antigen, substantial activity was recovered, as shown by Figure 2. A quenching of 16.5 per cent of the initial fluorescence is observed, and the reoxidized protein therefore had 20 per cent of the specific activity of the native fragment. A control experiment with reoxidized fragment I from nonspecific γ-globulin is also shown in Figure 2. No quenching of fluorescence is observed. The quenching observed with reoxidized antibody fragment cannot therefore be ascribed to a nonspecific binding resulting solely from the process of reduction and reoxidation. It should be noted that no detectable activity could be obtained from nonspecific fragment I even when it was reoxidized in the presence of a 100-fold excess of DNP-lysine, as is shown in Figure 3.

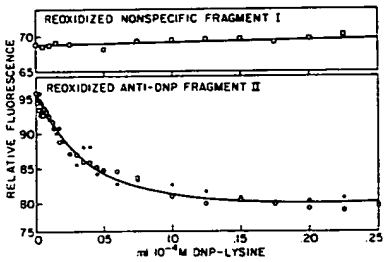

FIG. 2.—Duplicate titrations of reoxidized antibody fragment II with DNP-lysine. The upper curve represents a control experiment using fragment I, from nonspecific γ-globulin, which had gone through the same cycle of reactions as the antibody fragment.

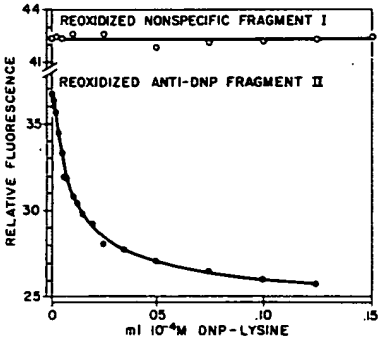

FIG. 3.—Titration of nonspecific fragment I and antibody fragment II after reoxidation in the presence of a 100-fold excess of DNP-lysine. (The hapten was removed from the reoxidized protein before the titration was carried out.)

The results of a number of assays of reoxidized antibody fragments are summarized in Table 2. It is seen that the soluble portion of the reoxidized protein had between 14 and 24 per cent of the activity of native protein in different experiments. When acount is taken of the fact that not all of the protein subjected to reduction is recovered in soluble form, the total recovery of activity was about 8 per cent in each experiment. Table 2 includes results obtained by Haber[8] with reduced and reoxidized antiribonuclease fragment. It is seen that his recovery of activity was somewhat higher than ours. He did not, however, perform a control experiment with reduced and reoxidized fragment I from nonspecific γ-globulin, so that it is possible that some of his recovered activity may represent nonspecific combination of antibody protein with ribonuclease, or with

lysozyme, which was also present in his assay mixture. We have observed such nonspecific combination between denatured and refolded nonspecific fragment I and bovine serum albumin coupled to polystyrene resin.[1]

TABLE 2

RECOVERY OF SPECIFIC ACTIVITY

	Recovery of soluble protein (per cent of native)	Activity expressed as per cent quenching of fluorescence	Recovery of Activity[a] (per cent of native) Based on amount of soluble protein	Recovery of Activity[a] (per cent of native) Based on total amount of protein[b]
Native fragment I	—	72	—	—
Reoxidized	30	17	24	7.1
Reoxidized	50	10	14	7.0
Native fragment II	—	84	—	—
Reoxidized	43	16.5	20	8.4
Native fragment II[c]	—	56	—	—
Reoxidized	50	9.5	17	8.5
Reoxidized anti-RNAse[d]	53	—	27	14.3
	58	—	20	11.6
Reoxidation in the presence of antigen[e]				
Native fragment II	—	84	—	—
Reoxidized	32	29	34	10.9

[a] The figures for recovery of activity are *minimal* figures, based on the assumption that combination of DNP-lysine with the active fragment always leads to 100% quenching of fluorescence. See discussion in text.

[b] The figures in this column are obtained by multiplying the recovery of soluble protein by the figures in the previous column. The possibility that some activity may reside in the insoluble portion of the product was not tested.

[c] This preparation was dialyzed against four changes of 30 vol of 6 *M* guanid ne hydrochloride, at 5°C, for a total of 36 hr, for the purpose of removing trace amounts of hapten which might be present in the original native protein.

[d] These data from Haber[4] are included for comparative purposes. The results are taken from experiments in which guanidine hydrochloride was used as unfolding agent.

[e] Reoxidized in the presence of a 100-fold molar excess of DNP-lysine, which was removed by dialysis against 6 *M* guanidine hydrochloride.

One of the experiments for which data are given in Table 2 represents an additional control experiment. We have pointed out that reduction and reoxidation were carried out in the absence of added antigen. It is, however, not possible to exclude the possibility that the native antibody fragment contains some residual 2,4-dinitrophenol not completely removed during the preparative procedure. (Contamination to the extent of about 5 per cent would not give sufficient absorbance to be detected by spectral measurement.) If such residual hapten is present, it could exert a directive influence during reoxidation and refolding. To eliminate this possibility, one sample of antibody fragment was dialyzed exhaustively against 6 *M* guanidine, such that any contaminant would have become diluted 800,000-fold. It was then reduced, reoxidized, and refolded in the usual manner. The product had a somewhat lower activity than the reoxidized fragments which had not been subjected to dialysis. However, native antibody fragment subjected to such lengthy dialysis against guanidine itself loses considerable activity. The table shows that the recovered activity, when compared to similarly dialyzed native protein, is the same as is obtained in the other experiments described in the table. The presence of the antigen is clearly not required for recovery of activity.

It should be noted that the reoxidized antibody fragments which retain anti-DNP activity do so mostly with reduced affinity for the antigen. This is evident from the slope of the quenching curve shown in Figure 2, which is much lower than that of Figure 1. The amount of DNP-lysine which must be added to attain

saturation of antibody combining sites is several times the stoichiometric amount, whereas with native fragment only a small excess over the stoichiometric amount is needed. (This finding suggests the possibility that we may well have underestimated the recovery of active molecules in the calculations carried out in Table 2. When the binding of DNP-lysine to an active fragment is weak, it may not quench all of the fluorescence. The observed per cent quenching of fluorescence may thus be an incomplete measure of the number of active molecules.)

The explanation for the diminished affinity for antigen may lie in the known heterogeneity of antibody protein, even when directed against a single antigen.[17-19] Because of this heterogeneity, both polypeptide chains of native antibody fragments will consist of populations of different species. It may be speculated that all possible combinations of A piece with B chain are not equally active, so that separation and recombination, even without irreversible alteration in any individual chain, can lead to molecules which have the correct specificity, but reduced binding affinity for antigen. In the experiments described in this paper some of the polypeptide chains have been irreversibly altered (exposure to 6 *M* guanidine alone leads to some irreversible inactivation[1, 2]), so that the reoxidized product may contain not only mismatched pairs of native A piece and native B chain, but also molecules in which a native A piece is combined with altered B chain. Such molecules may well have the ability to combine with antigen (A chain alone retains that ability[15]), but will do so only weakly.

The foregoing speculative explanation is supported by studies on the recombination of A and B chains of whole antibody.[20-22] These studies have shown that antibody A chains combine equally well with nonspecific B chains as with antibody B chains, if recombination is carried out in the presence of both kinds of B chain and in the absence of antigen. The product obtained by combination with nonspecific B chains has, however, a much lower affinity for antigen. If antigen is present, there is a preferred combination with antibody B chains, as might be expected in a situation where both kinds of B chain are equally available, and thermodynamic forces alone govern the choice.

These studies suggest a further experiment: they indicate that a product of higher affinity for antigen should be obtained if reoxidation is carried out in the presence of the antigenic determinant. Figure 3 and the last entry in Table 2 report the result of such an experiment, and show that this effect indeed occurs. The total per cent quenching is increased, which may indicate a higher yield of active molecules, or more complete quenching per molecule on combination with hapten. The important aspect of the result is that much of the quenching curve is now nearly as steep as that of Figure 1, indicating the presence of antibody fragments with very high affinity for antigen.

Discussion.—In the experiments described in this paper, and in a similar study reported recently by Haber,[8] active antibody fragments have been completely unfolded, dissociated into their constituent polypeptide chains, and reduced so as to destroy all intrachain disulfide bonds. After refolding and reoxidation, in the absence of the antigenic determinant, a soluble protein was obtained. Parallel experiments with nonspecific fragment I indicate that this protein has the same physical, chemical, and antigenic characteristics as the native fragment. It was found to have from 11 to 24 per cent of the specific antibody activity of native

protein. In the studies reported by Haber[8] even higher recovery of activity was reported.

Although the yield of active reoxidized protein is relatively small (nearly 100 per cent recovery has been reported for ribonuclease and lysozyme), it should be pointed out that special pains were not taken to assure a high yield. For example, the insoluble portion of the product was discarded, although it could probably have been reworked to produce additional active protein. In any event, the yield of active protein is far greater than the yield which would have been expected on a statistical basis if reoxidation of disulfide bonds were to occur randomly. According to Fleischman *et al.*,[15] active antibody fragments contain six disulfide bonds, three within A piece, two within the B chain, and one linking the two chains. If these bonds were reduced and reoxidized randomly, only one molecule in 10,395 would return to its original structure.[1] If reoxidation occurs independently in each chain before recombination of the chains takes place, as is in fact likely, the number of intramolecular bonds within each chain would remain constant. One molecule in 1,575 would then be expected to return to its original form. The observed recovery of active molecules is thus more than 100-fold greater than expected on the basis of random formation of disulfide bonds.[23]

It is evident, therefore, that the information required to create antibody specificity survives when all noncovalent interactions are disrupted and all disulfide bonds are broken. The information must therefore lie in the amino acid sequence of the protein.

Our results have bearing on two important problems in protein biochemistry.

(*a*) The biosynthesis of proteins, as presently understood, consists of the building of linear polypeptide chains, of fixed length and amino acid sequence, directed by specific sequences of bases in nucleic acid molecules. It has been proposed, especially by Anfinsen and his collaborators,[24] that the folding and cross linking of these chains, and their association with each other (where it occurs), occur spontaneously, directed solely by thermodynamic forces. As support for this proposal, it has been shown[4–7] that several simple proteins can recover their native three-dimensional structure and biological activity, spontaneously and *in vitro*, after complete disruption of noncovalent and of disulfide bonds, i.e., after being returned to the condition in which they existed at the conclusion of the biosynthetic process. It is as yet by no means certain, however, that this principle applies quite generally to all proteins. Recovery of activity after unfolding and disulfide bond cleavage has not heretofore been convincingly demonstrated for any protein which consists of more than one polypeptide chain and has both intra- and interchain disulfide bonds. For insulin,[25] attempts to restore activity in this way have led to only 5–10 per cent yield of active protein, and this is not significantly above the expected yield on the basis of random recombination of disulfide bonds. The results of this and of Haber's work thus provide an important new example to which Anfinsen's principle is applicable.

(*b*) The mechanism by which specific antibodies are produced is as yet unknown. It must contain some features not generally present in the process of protein biosynthesis. Three kinds of speculative theories have been advanced. (1) Burnet,[26] Lederberg,[27] Szilard,[28] and Smithies,[29] for example, have proposed that there exist numerous genes which contain the information to synthesize antibody polypeptide

chains with numerous different sequences, and that the antigen is somehow able selectively to stimulate synthesis of those chains which will form antibodies against it. (2) Schweet and Owen[30] have proposed that antigens can induce the formation of many different amino acid sequences from a single gene by somehow subverting the transcription of the genetic message. (3) Pauling[3] and Karush[31] have proposed that the amino acid sequence does not contain the information required for antibody specificity, but that antigen-directed formation of disulfide or noncovalent bonds is responsible instead. Our data support theories of types 1 and 2 only, since no antibody activity could be detected in a sample of nonspecific fragment I even when it was reoxidized in the presence of 100-fold excess of the antigenic determinant. On the other hand, substantial activity was recovered from antibody fragment even when extreme measures were taken to exclude the presence of hapten during reoxidation.

The presence of excess hapten during reoxidation did affect the recovery of activity from reduced antibody fragment. The affinity of the recovered protein for the haptenic DNP group was markedly increased. It is likely that this effect resulted from an influence of hapten on the combination of refolded A piece with refolded B chain, rather than from an influence on reoxidation and refolding of the individual chains. A similar phenomenon could play a role in the *in vivo* production of antibodies, but only if a freshly synthesized A chain had a choice of many different B chains during the final assembly of the γ-globulin molecule.

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*NITROGEN FIXATION: HYDROSULFITE AS ELECTRON DONOR WITH CELL-FREE PREPARATIONS OF AZOTOBACTER VINELANDII AND RHODOSPIRILLUM RUBRUM**

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Nitrogen fixation with cell-free extracts of the aerobe, *Azotobacter vinelandii*, was recently reported.[1] Hydrogen gas, coupled via a hydrogenase and ferredoxin preparation from *Clostridium pasteurianum*, served as the electron donor, and low levels of ATP were supplied with an ATP-generating system. These experiments have established that O_2 *per se* is not required for fixation by the nitrogenase system of this aerobe and that the nitrogenase activity of Azotobacter fractions is stable in air. After the presence of nitrogenase activity in Azotobacter preparations was demonstrated, alternate electron donor systems were examined in an attempt to obviate the requirement for the hydrogenase and ferredoxin preparation and thus reduce the complexity of the system (particularly in view of the known hydrogen inhibition of fixation by intact cells).

This report describes the use of sodium hydrosulfite as the electron donor for nitrogen fixation by cell-free preparations of Azotobacter and gives the optimal conditions for measuring fixation activity. Evidence is presented that ammonia is the principal product of N_2 reduction. Manometric data demonstrating the presence of an ATP-dependent hydrogenase in the extracts are also presented.

Cell-free fixation by extracts of *Rhodospirillum rubrum* was observed by Schneider *et al.*[2] using the method of Carnahan *et al.* to prepare extracts of dried cells. Because the source of electrons for N_2 reduction was uncertain, and because difficulty was encountered in reproducing their results, the successful use of a compound known to function as an electron donor appeared to be a prerequisite for reproducible fixation reactions. A demonstration of the ability of hydrosulfite to serve as the electron donor for N_2 reduction by extracts of freshly harvested *R. rubrum* cells is included in this report.

Materials and Methods.—*Azotobacter vinelandii O* was maintained in liquid culture as previously described.[3] For enzyme isolation, 6-liter cultures were incubated for 16 hr at 30° in 10-liter solu-